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Nicotinic Receptors Underlying Nicotine Dependence:

Evidence from Transgenic Mouse Models

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Abstract

Nicotine underlies the reinforcing properties of tobacco cigarettes and e-cigarettes. After inhalation and absorption, nicotine binds to various nicotinic acetylcholine receptor (nAChR) subtypes localized on the pre- and post-synaptic membranes of cells, which subsequently leads to the modulation of cellular function and neurotransmitter signaling. In this chapter, we begin by briefly reviewing the current understanding of nicotine's actions on nAChRs and highlight considerations regarding nAChR subtype localization and pharmacodynamics. Thereafter, we discuss the seminal discoveries derived from genetically modified mouse models, which have greatly contributed to our understanding of nicotine's effects on the reward-related mesolimbic pathway and the aversion-related habenulo-interpeduncular pathway. Thereafter, emerging areas of research focusing on modulation of nAChR expression and/or function are considered. Taken together, these discoveries have provided a foundational understanding of various genetic, neurobiological and behavioral factors underlying the motivation to use nicotine and related dependence processes, which are thereby advancing drug discovery efforts to promote long-term abstinence.

Introduction

Nicotine is the primary active constituent in tobacco-containing products, which is responsible for maintaining smoking behavior in humans (Stolerman and Jarvis 1995). Recently, nicotine has also been formulated for vapor inhalation via e-cigarettes devices (Electronic Nicotine Delivery Systems, or ENDS; (St Helen et al. 2016)). Concomitant with a decrease in combustible tobacco cigarette use, use of e-cigarettes, especially among adolescents, has drastically risen in recent years (Wang et al. 2018). Indeed, from 2017-2018, there was a rapid increase in vaping prevalence among adolescents aged ~13-18 years old, with nicotine vaping rates translating to roughly an additional 1.3 million adolescent users in 2018 compared to 2017 (Miech et al. 2019). Although e-cigarettes may have value as a nicotine replacement strategy for current tobacco smokers (Hajek et al. 2019), the increasing patterns of e-cigarette use among adolescents has become of high concern and warrants further investigation. As well, among current smokers, some studies show that e-cigarettes are not liked as much as tobacco cigarettes (Strasser et al. 2016), and therefore, additional research is needed to determine the ability of e-cigarettes to accomplish nicotine replacement, harm reduction, or act as a quit aid (Rennie et al. 2016; Selya et al. 2018).

Chronic exposure to nicotine or nicotine-containing products is associated with detrimental health effects, including enhanced brain injury and/or stroke risk (Sifat et al. 2018), altered blood brain barrier permeability (Hawkins et al. 2004), promotion of tumor growth via nicotine and its carcinogenic metabolites cotinine, N'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK; (Ginzkey et al. 2013; Ginzkey et al. 2012; Jacob et al. 2009; Nakada et

al. 2012), early onset of menopause in women (Bellavia et al. 2016), among others. Of importance, recent research has found that e-cigarette smoke may be carcinogenic and lead to increased risk of lung and bladder cancer, as well as heart disease, due to DNA damage (Lee et al. 2018). Given the harmful health effects associated with chronic use of nicotine-containing products, understanding the mechanisms that drive nicotine use is essential.

Nicotine is an agonist at nicotinic acetylcholine receptors (nAChRs), the structure and function of which were discovered in the early 1980s using ligand-binding assays (Clarke et al. 1985; Patrick and Stallcup 1977). The nAChRs are members of a large family of cys-loop homologous receptors, which also includes muscle acetylcholine receptors (mAChRs), GABAA/C, glycine, and serotonin type 3 receptors (Miller and Smart 2010). The nAChRs are pentameric ion channels, whereby either homomeric or heteromeric subunits combine together to form a central pore (Zoli et al. 2015). The subunit composition and stoichiometry of nAChRs determine its unique pharmacological binding profile, as well as its susceptibility to desensitization (Picciotto et al. 2008).

Decades of research has uncovered important neural mechanisms that drive nicotine self-administration behavior. Specifically, nicotine engenders self-administration through activation of high affinity β2 subunit-containing (denoted $\beta 2^*$) nAChRs localized on dopamine-containing cell bodies within the ventral tegmental area (VTA; (Klink et al. 2001; Picciotto et al. 1998)), and by altering glutamatergic and GABAergic tone in the VTA (Mansvelder et al. 2002). The net result of nicotine-induced activation of nAChRs is increased levels of extracellular dopamine within the

nucleus accumbens (NAc; (Pontieri et al. 1996)), which is hypothesized to contribute to its reinforcing effects. Although activation of nAChRs can lead to motivated behavior, it is also through desensitization/inactivation that nAChRs can alter acetylcholine signaling and neuronal function (Colombo et al. 2013), which may also contribute to modulation of nicotine-motivated behavior. Importantly, both acetylcholine and nicotine lead to nAChR desensitization; however, nicotine leads to prolonged inactivation of these receptors, with a slower rate of recovery than the endogenous ligand (Giniatullin et al. 2005). Interestingly, α4β2 nAChR desensitization occurs following cigarette smoking, which is correlated with reductions in cigarette craving (Brody et al. 2006).

It is important to note that nAChRs are expressed neuronally, on both pre- and post-synaptic terminals as well as on post-synaptic somatodendrites (Albuquerque et al. 2009). Additionally, some nAChRs (e.g., homomeric α7) are localized on astrocytes and microglia in the brain (Graham et al. 2003; Jensen et al. 1997; Noda and Kobayashi 2017; Shen and Yakel 2012; Shytle et al. 2004), which have important functions at glutamatergic neuronal synapses that impact synaptic plasticity (Wang et al. 2013). Subunit composition of nAChRs in the brain can vary depending on region and cell-type specific localization, the topography of which continues to be studied (Gaimarri et al. 2007; Gotti et al. 2006; Hendrickson et al. 2013), and is important in understanding neurobehavioral processes modulated by nAChRs.

In this chapter, we describe the current state of the knowledge regarding nAChR subtype expression in a brain region- and pathway-specific manner as it relates to nicotine dependence

and comorbid pathologies. To accomplish this goal, we address current tools in the field that have allowed for exploration of the role of nAChR function and expression in addiction-related processes, with a focus on findings garnered from transgenic mouse models. As well, we illuminate novel areas of research focusing on modulating nAChR expression and/or function, which may have important implications for nicotine dependence processes. Given the evolving landscape of nicotine-containing product use (Fowler et al. 2017), a better understanding of the neural processes underlying the motivation to use nicotine is needed to enhance drug discovery efforts to promote cessation from nicotine-containing products.

nAChR Function and Signaling

As noted above, the nAChR receptor composition plays an important role in response to pharmacological agents. When an agonist (e.g., acetylcholine or nicotine) is bound to nAChRs, the receptors are first activated and then can desensitize, followed by recovery once the agonist is unbound. The EC50 value, which represents nAChR activation for the concentration of agonist producing half-maximal response amplitude, varies based on subunit composition. For example, the acetylcholine EC50 value is 513 for rat α7 (Papke and Porter Papke 2002), but is 14 for rat α3β4 (Bohler et al. 2001). Conversely, the measure for desensitization, or the concentration of agonist required to reduce the amplitude of the response by 50%, is termed the IC50. Based on subunit composition, desensitization-induced inhibition of receptors can vary when activated by the same agonist; for instance, the rat α4β2 IC50 with nicotine is <0.01 (Paradiso and Steinbach 2003), whereas rat α7 is 1.3 (Fenster et al. 1997; Giniatullin et al. 2005). These values represent different rates of activation and inhibition, which have profound effects on nAChR modulation of

neuronal function. It should be noted that the same receptor subtype can desensitize at different rates based on the agonist present. For example, rat α7 IC50 value is 1.3 for nicotine, but is >10,000 for acetylcholine (Fenster et al. 1997; Papke and Porter Papke 2002). This is important because recovery rates are dependent upon the agonist present, with recovery rate from nicotine taking longer than acetylcholine in some cases (e.g., α4β2; (Paradiso and Steinbach 2003)). Given the important role of nAChR subtypes such as α4β2 in the reinforcing effects of nicotine (Changeux et al. 1998) and in modulating dopamine release (Mansvelder and McGehee 2002), different rates of desensitization and recovery of different nAChRs likely play key roles in the neural circuitry underlying nicotine addiction.

There are generally two binding sites for neuronal heteromeric nAChRs, each of which is formed by a pocket between subunits extracellularly at the ligand-binding N-terminal domain (Karlin 2002; Sine et al. 2002). Neuronally, α7 nAChRs are mainly homomeric and have five potential binding sites between the α subunits (Drisdel and Green 2000). Recently, additional binding sites for heteromeric nAChRs have been identified which are dependent on subunit composition (Jain et al. 2016). When a ligand is bound, the channel opens within microseconds (Albuquerque et al. 2009), indicative of the rapid responsivity of these channels. A sequence of events occurs to alter the conformational state of the channel in order to open. Through computer generated modeling, it has been determined that when acetylcholine or nicotine is bound, hydrogen bonds among amino acids rearrange near the binding pocket. Subsequently, the C-loop moves toward the central pore, which then allows the Cys-Cys pair to interact with the bound ligand and results

in the ligand being trapped deep within the pore between the subunits (Gao et al. 2005; Hansen et al. 2005).

As mentioned above, neuronal nAChRs can be expressed somatodendritically, pre-synaptically, or post-synaptically (Broide and Leslie 1999; McGehee et al. 1995; Wonnacott 1997). Nicotine binds to nAChRs located in the brain, which have identified subunits of α2-7, and β2-4 (Boulter et al. 1987; Couturier et al. 1990; Picciotto et al. 2008). Somatodendritically-expressed nAChRs play a modulatory role in the neurotransmission of other systems in response to nicotine (Wonnacott et al. 2006), such as dopamine (Nisell et al. 1994). nAChRs that are expressed somatodendritically and modulate dopamine release appear to contain α6 and β3 subunits, and differ in pharmacological response to nicotine and epibatidine compared to those expressed on terminals in the striatum (Reuben et al. 2000). Presynaptic nAChRs are also important in modulating neurotransmitter release. For instance, α7 nAChRs are Ca^{2+}-permeable, rapidly desensitize following activation and are expressed on many cell types including at glutamatergic terminals in brain regions such as the hippocampus and NAc (Fabian-Fine et al. 2001; Kaiser and Wonnacott 2000). Thus, nAChRs can enhance release of neurotransmitters from synaptic terminals, and may provide a feedforward mechanism by which cholinergic signaling gates neurotransmitter release. Post-synaptically, nAChRs modulate many functions within the brain, including the flow of auditory information in the thalamus. Specifically, β2-containing heteromeric nAChRs are located on neurons within the medial geniculate body receive cholinergic input from the pontomesencephalic tegmentum, and these receptors undergo an age-related decline in their expression and function (Sottile et al. 2017). As well, postsynaptic

nAChR subunits such as α6, α7, β2, and/or β4 within the laterodorsal tegmentum, undergo changes in subunit composition due to age, which results in differential nicotine-induced neuronal excitability (Christensen and Kohlmeier 2016; Kaneda 2017; Kaneda et al. 2016; Shinohara et al. 2014; Taoka et al. 2016).

For decades, research has shown that chronic exposure to nicotine significantly alters expression and function of neuronal nAChRs (Fenster et al. 1999a; Fenster et al. 1999b; Gentry and Lukas 2002; Quick and Lester 2002). One nAChR subunit heavily involved in nicotine-induced striatal dopamine release and nicotine self-administration behavior, β2 (see a more thorough description below), is upregulated and desensitized after chronic nicotine exposure, as measured via binding assays in intact oocytes (Fenster et al. 1999b). Following chronic activation due to nicotine, different subunit-containing nAChRs appear to desensitize at different rates, which is thought to underlie their ability to modulate different neurotransmitter systems. Further, changes in receptor expression with chronic nicotine appears to be cell type and nAChR subtype dependent (Benwell et al. 1988; Lallai et al. 2019; Marks et al. 1992; Perry et al. 1999). Both neuronal and non-neuronal cholinergic signaling involves some of the same subtypes of nAChRs and are associated with pathologies such as lung cancer (Mucchietto et al. 2016). One such subunit is α5, which is expressed in non-neuronal tissues (Chini et al. 1992) such as lung, pancreas, stomach, and gliomas (Jia et al. 2016; Yoshikawa et al. 2005; Zia et al. 1997), and may mediate nicotine-induced lung cancer cell proliferation (Ma et al. 2014; Sun and Ma 2015). This subunit forms functional complexes with α4β2 or α3β4 subunits, and polymorphism of the human α5 gene, *CHRNA5,* is associated with nicotine dependence and lung cancer (Bierut et al. 2008; Chen et al.

2009; Saccone et al. 2009; Saccone et al. 2007). Importantly, the variant of α5, characterized by a change in the 398th amino acid from aspartic acid to asparagine (D398N), has been associated with a reduction in the function of the human α3β4α5 nAChR (George et al. 2012), which has important implications for smoking cessation outcomes as well as other health pathologies.

nAChRs Mediating Nicotine Reinforcement

Genetically modified mouse models have allowed for the interrogation of receptors and circuits underlying complex behaviors. In the tobacco and nicotine field, groundbreaking studies by Picciotto, Changeux and colleagues (Picciotto et al. 1995; Picciotto et al. 1998) have provided an important foundation for the further progression of these animal models. Beginning with knockout mice, subsequent approaches have incorporated various genetic and technical tools to achieve more select manipulation of target protein or neurotransmitter function. These advances include, but are not limited to, humanized knockin genes, modified receptors, cre driver lines with floxed viral approaches, optogenetic and chemicogenetic expression of receptors in a cell type specific manner, promotor driven fluorescent reporter lines, and most recently, CRISPR-Cas9 directed genetic modifications. Findings derived thus far from such approaches within each circuit are discussed in the following paragraphs.

Mesolimbic Pathway

The positive rewarding effects of nicotine involve the brain's mesolimbic pathway (Kenny and Markou 2005; Rice and Cragg 2004), consisting of dopaminergic projections from the VTA. The VTA integrating circuits and projection regions contain various nAChR subtypes expressed on

dopaminergic, glutamatergic and GABAergic neurons (Charpantier et al. 1998; Klink et al. 2001; Mameli-Engvall et al. 2006; Mansvelder and McGehee 2002). For instance, inhibitory GABAergic projections from the rostromedial tegmental nucleus (RMTg) express terminal $\alpha4\beta2$* nAChRs. VTA dopaminergic cells projecting to both the NAc and prefrontal cortex (PFC) express $\alpha4\alpha6\beta2$, $\alpha4\beta2$, and $\alpha6\beta2$ nAChRs, allowing for regulation of dopamine signaling through either somatic or presynaptic expression. These VTA dopaminergic neurons may also co-express glutamate or GABA, and it has been recently shown that heteromeric nAChRs mediate excitatory signaling in the dopaminergic-glutamate co-expressing cells (Yan et al. 2018). Within the NAc, the dopaminergic terminal nAChRs become activated by cholinergic interneurons and modulate dopamine's activation of GABAergic medium spiny neurons expressing dopamine D1 or D2 receptors. Intra-VTA glutamatergic circuits also appear to modulate GABAergic signaling via axoaxonic connections onto RMTg terminals. Further, glutamatergic projections from other brain regions, such as the PFC and subiculum, express presynaptic $\alpha7$ nAChRs and have been found to terminate on the soma of dopaminergic neurons. Moreover, expression of the $\alpha2$, $\alpha5$ and $\beta3$ nAChR subunits have also been localized within the VTA. Together, this complicated pattern of nAChR expression makes defining the specific subtype contribution to nicotine reward and reinforcement challenging. However, significant advances have been made in this regard.

Initial studies in knockout mice have supported pharmacological findings implicating nAChRs expressing the $\beta2$ nAChR subunit in mediating reward- and reinforcement-related processes. In the striatum, nicotine application induces a robust increase in dopamine release, which can be blocked by administration of the nAChR antagonist mecamylamine (Mifsud et al. 1989). However,

this nicotine-mediated increase in dopamine release was absent in the striatum of mice lacking the β2 nAChR subunit (Picciotto et al. 1998). To examine the involvement of this subunit on nicotine reinforcement, mice were assessed in an intravenous nicotine self-administration protocol, a technique with high translational validity to patterns of nicotine consumption in humans. Interestingly, while the wildtype mice exhibited sustained nicotine self-administration behavior, the β2 knockout mice did not self-administer nicotine (Picciotto et al. 1998). A further study revealed similar findings with a lack of sustained self-administration behavior in the absence of the β2 nAChR subunit with nicotine infusions directly into the VTA (Maskos et al. 2005). More recently, viral mediated re-expression of the β2 nAChR subunit in the VTA of the knockout mice was shown to 'rescue' the behavioral phenotype, in which this site-specific re-expression led to the mice acquiring nicotine self-administration (Orejarena et al. 2012). Additional support from studies with β2 knockout mice demonstrate that the β2* nAChR is necessary for the formation of a conditioned place preference to a nicotine-paired environment and the discriminative stimulus properties of nicotine (Shoaib et al. 2002; Walters et al. 2006). In a cutting edge approach, Mourot and colleagues used a viral technique to express light-controllable β2* nAChRs in the VTA, and during light exposure, the VTA β2* nAChRs became inhibited, which thereby was sufficient to prevent the formation of a nicotine-induced conditioned place preference (Durand-de Cuttoli et al. 2018).

In addition to the β2 subunit, lack of sustained nicotine self-administration has also been found in mice with knockout of the α4 and α6 nAChR subunits, and importantly, the behavioral phenotype could be restored with reexpression of these subunits in the VTA of each respective

knockout line (Exley et al. 2011; Maskos et al. 2005; Picciotto et al. 1998; Pons et al. 2008). Further, dopaminergic neuron specific deletion of the α4 subunit was found to prevent the formation of a nicotine-mediated conditioned place preference (McGranahan et al. 2011). In a complementary approach, transgenic α4 and α6 nAChR hypersensitive knockin mice were generated, in which a single point mutation renders the receptor subtype more responsive to nicotine. For the α4 subunit, this genetic modification led to an enhancement of the rewarding effects of nicotine, as assessed with conditioned place preference (Tapper et al. 2004), and for the α6 subunit, mice exhibited a potentiation of nicotine-mediated locomotor effects and increased glutamatergic transmission with VTA neurons (Berry et al. 2015). As further evidence for these specific receptor subtypes, pharmacological administration of the relatively selective α4β2 nAChR antagonist, DHβE, also decreased nicotine self-administration in rats (Corrigall and Coen 1989; Harvey et al. 1996; Watkins et al. 1999). These findings are paralleled by studies demonstrating that DHβE attenuates the stimulatory effects of nicotine on brain reward systems (Harrison et al. 2002). Together, these findings support the notion that α4β2 and/or α4α6β2 nAChRs on dopaminergic circuits in the VTA mediate the reinforcing properties of nicotine.

The involvement of the α7 nAChR in nicotine dependence has been somewhat controversial. As noted above, glutamatergic axons containing presynaptic α7 nAChRs terminate on the soma of dopaminergic neurons in the VTA, suggesting a regulatory role for downstream dopaminergic signaling. Initial pharmacological studies demonstrated that administration of the α7 selective antagonist, methyllycaconitine, attenuates nicotine self-administration in rats (Markou and Paterson 2001), a finding that was further substantiated with site-specific VTA injections in

wildtype mice (Besson et al. 2012). However, studies in α7 nAChR knockout mice failed to find differences with intravenous nicotine self-administration and nicotine-mediated conditioned place preference compared to wildtype littermates (Pons et al. 2008). However, more recently, Granon and colleagues (2012) were able to establish a dose-dependent effect with intra-VTA nicotine self-administration, in which the α7 nAChR knockout mice exhibited decreased self-administration at a low, but not high, nicotine dose. Further, when administered a peripheral injection of nicotine, nicotine-induced dopamine outflow in the NAc was sustained over a longer period of time in the α7 knockout mice (120 min), as compared to the wildtype mice (15 min) (Besson et al. 2012). In consideration of α7 nAChRs' presynaptic circuit localization, lower affinity for nicotine, and rapid recovery from desensitization, the receptor's effects on the mechanisms underlying nicotine reinforcement appear to be more nuanced.

Habenulo-Interpeduncular Pathway

As a drug of abuse, nicotine is distinctive in that the aversive properties appear to sharply contrast the rewarding properties of the drug, thereby limiting the range of doses that promote reinforcement and drug consumption. Nicotine's aversive effects are mediated by the medial habenula (MHb), a brain structure that directly projects to the interpeduncular nucleus (IPN). The MHb-IPN circuit has been characterized as containing the densest expression of cholinergic fibers and various nAChR subunits within the brain, including the α5, α3 and β4 nAChR subunits (Marks et al. 1992; Villani et al. 1983). The aversive signaling of this circuit has been demonstrated in several studies with genetically modified rodents. For instance, α5 nAChR subunit knockout mice exhibit a high level of motivation to consume large quantities of nicotine, and viral mediated re-

expression of α5 subunits within this pathway restores nicotine intake to wildtype levels (Fowler et al. 2011). In addition, while wildtype mice exhibit inhibitory motivational effects at high doses of nicotine, the α5 nAChR knockout mice continue to exhibit reward-related effects, as assessed with both conditioned place preference and intracranial self-stimulation (Fowler et al. 2013; Jackson et al. 2010). The conclusions drawn from the knockout mice are supported by complementary studies using viral mediated knockdown of the α5 nAChR subunit in rats, in which decreased expression of α5 nAChR subunits selectively in the habenula similarly increase nicotine intake and also decreased the inhibitory effects of higher nicotine doses on the activity of the brain reward circuitry (Fowler et al. 2011). Presynaptic α5* nAChRs on MHb terminals appear to facilitate glutamate release from cholinergic and glutamatergic coexpressing axons in the IPN (Fowler et al. 2011; Girod and Role 2001), which is thought to mediate this effect. Further, chronic nicotine appears to mitigate the activation of a subpopulation of α5-expressing neurons in the IPN, which subsequently provide negative feedback onto habenular terminals and mitigate nicotine reward, as assessed with conditioned place preference (Ables et al. 2017). The presence of the α5 nAChR subunit in α4β2, α3β2 and α3β4 nAChR receptors have been shown to alter nicotine binding and/or desensitization kinetics *in vitro* (Ramirez-Latorre et al. 1996; Wang et al. 1996), and all of these subtypes are expressed in the MHb-IPN pathway. Furthermore, the β4 nAChR subunit has also been shown to mediate aversive processing for nicotine. Under conditions of β4 nAChR subunit over-expression, mice consume less nicotine solution (Frahm et al. 2011), thereby suggesting that an α5β4* nAChR subtype may underlie an inhibitory motivational signal for nicotine in the MHb-IPN pathway. These findings in mouse models are further supported by human genome wide association studies demonstrating that allelic

variation in the *CHRNA3-CHRNA5-CHRNB4* gene cluster, which encodes α3, α5 and β4, respectively, increases vulnerability to developing tobacco dependence (Bierut et al. 2008; Kapoor et al. 2012; Wang et al. 2009). Recently, the non-synonymous SNP in the α5 gene that has been implicated in nicotine dependence in humans was inserted into the genome of rats to generate a transgenic humanized α5SNP model (Forget et al. 2018). The behavior of the α5SNP rat closely parallels the mouse knockout model, in which greater levels of nicotine are self-administered at high doses. In addition, an increase in nicotine-induced reinstatement was found in the α5SNP rats (Forget et al. 2018), suggesting a role for this genetic variant in relapse-related behavior.

nAChRs in Other Aspects of Nicotine Dependence

Nicotine Enhancement of Cue Association

Nicotine administration has been shown to enhance the acquisition of certain learned behaviors, such as contextual fear conditioning and trace cued fear conditioning. These findings may underlie nicotine's cue-related conditioning effects with drug use, in that later exposure to the cue during abstinence may promote drug relapse. Nicotine's enhancing effect on contextual fear conditioning is prevented in mice with knockout of the β2, but not α7, nAChR subunit (Davis and Gould 2007; Portugal et al. 2008). These effects likely involve the hippocampus since systemic or site-specific hippocampal administration of the β2 nAChR antagonist DHβE mitigates contextual fear learning in wildtype, but not β2 subunit knockout, mice (Davis and Gould 2007; Portugal et al. 2008). An enhancement of nicotine-mediated cued, but not trace or contextual, fear conditioning was also found in female, but not male, α2 nAChR subunit knockout mice (Lotfipour

et al. 2013). Interestingly, mice with a hypersensitive α2* nAChR exhibit impaired contextual fear conditioning, an effect which could be rescued with pretreatment of nicotine (Lotfipour et al. 2017).

Nicotine Withdrawal

Following chronic nicotine administration, wildtype mice exhibit a range of behaviors indicative of the withdrawal state, including somatic signs (such as shaking, paw tremors, writhing), increased anxiety-like behavior in the elevated plus maze, increased brain reward thresholds, learning deficits in a contextual fear conditioning paradigm, and development of a conditioned place aversion to a withdrawal-associated environment. Studies with the β2 knockout mouse indicate that β2* nAChRs are involved in withdrawal-related anxiety-like behavior and conditioned place aversion, but not in the expression of somatic withdrawal signs (Jackson et al. 2008; Salas et al. 2004). Further, α7 nAChRs have been implicated the initial expression of withdrawal symptomology, including anhedonia and somatic signs, but the α7 subunit knockout mice do not differ from wildtype mice at later time points (e.g., 24+ hours) (Grabus et al. 2005; Salas et al. 2007; Stoker et al. 2012). Moreover, decreased somatic withdrawal signs have been found in α2, α5 and β4 nAChR subunit knockout mice, as compared to their respective wildtype littermates (Lotfipour et al. 2013; Salas et al. 2004; Salas et al. 2009). Interestingly, all of these subunits exhibit selectively dense expression in the MHb-IPN pathway, which has also been specifically implicated in somatic aspects of nicotine withdrawal. Administration of the general nAChR antagonist mecamylamine into the MHb-IPN pathway is sufficient to precipitate withdrawal, whereas injections into the cortex, VTA or hippocampus are ineffective (Salas et al.

2009), and re-exposure to nicotine during withdrawal results in increased activity of MHb and IPN neurons (Arvin et al. 2019; Gorlich et al. 2013). Further, injections of antagonists for $\alpha4\beta2$* or $\alpha6\beta2$*, but not $\alpha3\beta4$*, nAChRs in the MHb decrease the expression of anxiety-related behavior under conditions of nicotine withdrawal in mice (Pang et al. 2016). Together, these findings suggest that nAChRs are involved in various aspects of nicotine withdrawal based on their localization and expression patterns within the brain.

Modulators of nAChRs influencing expression and function

The expression and function of nAChRs may be modulated at various points from protein translation to membrane insertion to subsequent function. Early receptor binding studies in humans found increased expression of nAChRs in chronic smokers (Benwell et al. 1988; Perry et al. 1999), suggesting a change in cellular activation following prolonged nicotine exposure. Given that chronic agonist receptor activation typically results in receptor downregulation, this finding was unexpected, although it was also evidenced in more controlled rodent studies (Marks et al. 1983; Marks et al. 1992). The likely mechanism underlying receptor upregulation was recently elucidated as it was found that nicotine and nAChR ligands can act as 'chaperones' for $\alpha4$ and $\beta2$ nAChR subunits (Henderson et al. 2014; Kuryatov et al. 2005; Srinivasan et al. 2011), thereby allowing for increased expression of the high affinity nAChR subtype in the membrane. As the nAChR subunit protein is translated in the endoplasmic reticulum, the chaperone mechanism is thought to facilitate transport by promoting the trafficking of the protein to the plasma membrane, and subsequent insertion of the assembled nAChR.

Intracellular proteins have also been shown to stabilize nAChR subunits in the endoplasmic reticulum and regulate subunit assembly into specific nAChR subtypes, resulting in either an increase or decrease in nAChR subtype-specific membrane expression (Dau et al. 2013; Wanamaker and Green 2007). For instance, α7 nAChRs are selectively targeted to the dendritic membrane by Ric-3, thus facilitating receptor expression (Alexander et al. 2010). In contrast, members of the Ly-6/neurotoxin gene superfamily, which includes lynx1 and lynx2, have been demonstrated to decrease receptor expression by acting as inhibitory chaperones during protein translation and trafficking, and moreover, lynx proteins also bind directly to the extracellular face of nAChRs on the cell membrane, resulting in a decrease in ligand binding efficiency and increase in the desensitization rate for nAChRs containing the α4, α3, α5, α7, β2, and/or β4 subunits (George et al. 2017; Ibanez-Tallon et al. 2002; Lyukmanova et al. 2011; Miwa et al. 1999; Nichols et al. 2014). In the cortex, Lynx1 is expressed in both glutamatergic and GABAergic neurons, whereas Lynx2 has been mainly localized in glutamatergic neurons (Demars and Morishita 2014). Lynx1 also appears to exhibit preferential binding affinity to the α:α interface, which would allow for increased interaction with the stoichiometry present in the lower sensitivity $\alpha4_3\beta2_2$ nAChRs (Nichols et al. 2014). In addition to intracellular proteins, other endogenous factors may interact with nAChRs to modulate function. For instance, estradiol has been shown to bind to the C-terminal tail of the α4 subunit to potentiate the activation of α4* nAChRs in the presence of acetylcholine, an effect that was selective for α4 as differences were not found with the α3 subunit (Curtis et al. 2002). More recently, phosphorylation sites have been identified on $\alpha4\beta2$* nAChRs, suggesting a direct role for the receptor in mediating calcium/calmodulin-dependent protein kinase II and protein kinase A intracellular signaling (Miller et al. 2018). Together, these

nAChR subtype specific interactions, along with cell type-specific expression patterns, may allow for selective modulation of various aspects of cholinergic signaling, thereby permitting each endogenous modulator to differentially regulate neural processes.

Beyond nicotine dependence

Although heavily involved in processes of nicotine dependence, nAChRs have also been implicated as mechanisms underlying other disease states, including Alzheimer's Disease (AD; (Lombardo and Maskos 2015)), schizophrenia (specifically, α7; (Jones 2018)), Parkinson's Disease (PD; (Jurado-Coronel et al. 2016)), overeating/weight gain (Shariff et al. 2016), among others. Discovery of these mechanisms has led to multiple Phase II clinical trials for nAChR compounds that have pro-cognitive effects (although many of these attempts have failed, see (Lewis et al. 2017)). Varenicline, a full agonist at α7 and a partial agonist at α4β2 nAChRs, is prescribed as a smoking cessation agent but also has efficacy in decreasing sucrose consumption and producing pro-cognitive effect in rodent models (Potasiewicz et al. 2018; Shariff et al. 2016). Interestingly, varenicline may improve cognitive function in patients with schizophrenia (Shim et al. 2012). In AD, medications have been developed that inhibit breakdown of the enzymes that metabolize acetylcholine (inhibition of acetycholinesterase and/or butyrylcholinesterase), such as Donezepil (Aricept) or Rivastigmine (Exelon). Additionally, drug development efforts have included compounds that act as positive allosteric modulators at α7 nAChRs in addition to AChE inhibition, including Galantamine (Reminyl). Galantamine slows progression of plaque formation preclinically (Bhattacharya et al. 2014), and has shown efficacy in improving cognition and global functioning in patients with AD (Deardorff et al. 2015). Although statistically significant, these

benefits are modest and thus additional drugs are needed. Taken together, these studies illustrate a need for refinement of medications that target nAChRs for indications beyond nicotine dependence.

Conclusions

Since the mid-1990's, significant advances have been made with transgenic animal models to allow for better interrogation of specific nAChRs and circuits underlying nicotine dependence. Studies have built upon prior findings to reveal integral roles for various subunits in the mechanisms underlying nicotine's actions in the brain, with relevance to addiction. The $\alpha4\alpha6\beta2$* nAChRs in the mesolimbic pathway appear to be important in mediating the reinforcing properties of nicotine, whereas the $\alpha5$ and $\beta4$ nAChR subunits in the MHb-IPN mitigate the aversive properties of higher nicotine doses that thereby limit drug intake. In addition to these effects on drug consumption, nAChRs have also been implicated in other aspects of the dependence processes, including withdrawal, cue-associated learning, and psychiatric comorbidity. This foundation holds the promise to provide the field with a basis for new discoveries to formulate more efficacious therapeutics. For instance, in consideration of the involvement of the $\alpha4\beta2$* nAChRs in nicotine reinforcement, it is perhaps not surprising that varenicline has similar or greater effectiveness in promoting smoking cessation compared to nicotine replacement therapy and other approved therapeutics, such as bupropion (Gonzales et al. 2006). Drug development efforts are also focused on modulating the MHb-IPN circuit to enhance nicotine-mediated aversion and thus decrease further drug intake (Fowler and Kenny 2014; Jin et al. 2014). For instance, GLP-1 receptors have been shown to alter nicotine intake via

modulation of the MHb-IPN circuit (Tuesta et al. 2017), and a GLP-1 receptor agonist, liraglutide, is currently being tested for smoking cessation in a clinical trial (Ashare 2019). In another approach to minimize nicotine entry into the brain, NicA2-J1 has been developed as a reengineered nicotine-degrading enzyme (Kallupi et al. 2018). Interestingly, while NicA2-J1 does not appear to induce significant differences in nicotine intake, decreased withdrawal and relapse-related behaviors were found in rats (Kallupi et al. 2018). Therefore, the field will certainly continue to advance by better defining the various genetic, behavioral and biological mechanisms underlying addiction so that long-term abstinence can be readily achieved by those seeking to quit tobacco and e-cigarettes.

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